Adenoid Cystic Carcinoma: Participation of Urokinase-type Plasminogen Activator (uPA) and its Receptor (uPAR) in Tumor Invasion

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Abstract: Adenoid cystic carcinoma (AdCC) is one of the most common malignant tumors of the salivary glands and has unique clinical features and behavior. AdCC grows slowly, but spreads relentlessly into adjacent tissues, with a proclivity for invading nerve and endothelial sheaths. Moreover, the frequency of recurrence and distant metastasis of AdCC is very high. In vivo and in vitro, AdCC produces a large amount of extracellular matrix (ECM), including basement membrane (BM) components, elastin, and mucopolysaccharides. The accumulation of ECM components in intercellular spaces results in the formation of a pseudocyst, which is the characteristic architecture of AdCC. AdCC cells degrade considerable amounts of mesenchymal-elaborated ECM through the urokinase-type plasminogen activator (uPA)-plasmin system. By contrast, tumor-produced ECM is resistant to degradation, because it contains plasminogen activator inhibitor type 1 (PAI-1). The migration response of AdCC cell lines to ECM, especially type I and type IV collagens, is much stronger than that of oral squamous cell carcinoma (SCC) cell lines, while both cell types generally show similar patterns of integrin subunit expression. The AdCC cell response to collagens is largely and exclusively inhibited by anti-α2 integrin antibody. Surface uPA receptor (uPAR) expression by AdCC cell lines is greater than that by SCC cell lines and increases in response to collagen stimulation. This is accompanied by the assembly of numerous focal adhesions, consisting of the adapter proteins uPAR, α2 integrin, vinculin, and paxillin. A role for uPAR in cell migration and assembly of adaptor proteins was also demonstrated by transfecting AdCC cells with an antisense uPAR RNA, which strongly reduced both responses. Therefore, the proclivity of AdCC cells to migrate to type I and IV collagens might be due to the overexpression of uPAR, which also plays a key role in focal adhesion assembly. In conclusion, the invasiveness of AdCC cells might be regulated by the interaction of uPA-uPAR with integrin.

Key words: adenoid cystic carcinoma, recurrence, invasion and metastasis, urokinase-type plasminogen activator (uPA), uPA receptor, plasminogen activator inhibitor type 1, matrix degradation, integrins, cell migration, focal adhesion

1. Introduction

Adenoid cystic carcinoma (AdCC) is one of the most common malignant tumors of the salivary glands. The tumor grows slowly, but spreads relentlessly into adjacent tissues, so recurrence is common and distant metastasis, especially to the lungs, is very frequent. Since AdCC is resistant to radiotherapy and chemotherapy,
effective forms of treatment are required. Histologically, the tumor is characterized by a cylindrical or cystic stroma surrounded by anastomosing cords of epithelial tumor cells. The cystic stroma and the interstitium surrounding the tumor islands are often hyalinized and contain various substances, including collagen-like fibers, elastin, basement membrane constituents, and mucopolysaccharides.

Invasion and metastasis are characteristic features of malignant tumors. Although the molecular mechanisms underlying metastasis are complex, it is clear that tumor cells must migrate through the extracellular matrix (ECM) in order to invade local tissues and metastasize to distal sites. Migrating cells use both adhesion molecules and proteolytic enzymes to regulate their interaction with and response to the ECM, and cooperation between these components operates at several levels: integrin signaling induces proteases, proteases co-localize with integrins, and proteases regulate the interface between integrins and the cytoskeleton. A protease system intimately connected to integrins comprises urokinase-type plasminogen activator (uPA), the uPA receptor (uPAR), and plasmin.

The plasminogen activation system, which is made up of plasminogen activators, their inhibitors, plasminogen, and the respective cell-surface-binding proteins and receptors, plays a central role in cell invasion. Along with tissue-type plasminogen activator, uPA promotes the formation of plasmin, the key protease in fibrinolysis. Plasmin activates matrix-metalloproteinases, which constitute a proteolytic system for cell migration and tissue remodeling. Together, these proteases facilitate cell invasion by (1) activating latent growth factors or releasing them from their ECM-binding sites, and (2) degrading a variety of proteins in the ECM. Different plasminogen activator inhibitors (PAIs) neutralize plasminogen activator, primarily fast-acting PAI-1. PAI-1 is a serine protease inhibitor, but, unlike other proteases, its active conformation is stabilized by high-affinity binding to ECM-associated vitronectin. In addition to producing uPA, malignant cells also synthesize the uPA inhibitor PAI-1, thereby regulating PA activity levels in the cellular microenvironment. Therefore, tumor-cell-mediated proteolysis may be controlled by the fine regulation of tumor-cell-secreted proteases and protease inhibitors.

uPA binds to a glycosyl phosphatidylinositol (GPI)-anchored uPAR, which consists of three homologous domains (D1, D2, and D3). The binding of proteins such as uPA, vitronectin, and PAI-1 to uPAR involves a direct interaction with D1, but requires the integrity of the full-length receptor. uPA bound to uPAR exhibits enhanced proteolytic activity and directly activates plasminogen and matrix metalloproteinases, which, in turn, enhance ECM degradation. The receptor-binding domain of uPA is located in the amino-terminal fragment (ATF; residues 1-135) of the uPA molecule and does not involve the protease domain. Independent of its proteolytic function in matrix degradation, the uPA-uPAR interaction mediates several signaling events, regulates cell adhesion on vitronectin-coated surfaces, modulates integrin activity, triggers cell migration, and is strongly correlated with the metastatic potential of various tumors.

Integrins are ubiquitous, heterodimeric transmembrane receptors that anchor the cell to the ECM and the cytoskeleton to the plasma membrane. Integrin receptors mediate cell adhesion, migration, and bidirectional signal transduction via interactions with ECM proteins. In particular, the ECM-integrin interaction leads to the reorganization of the actin cytoskeleton, initiates signal transduction cascades, and coordinates the response to growth factors. Recent work points to important interactions between integrins, cytoskeletal components, and signaling molecules.

Focal adhesions are specialized cell adhesion sites that transduce signals from the ECM intracellularly. The assembly and disassembly of focal adhesions result in the formation and displacement of cellular stress fibers. On ligand activation, transmembrane ECM receptors, such as integrins, cluster at focal adhesions, which are physically connected to the cytoskeleton by stable and transient interactions. A characteristic property of most focal adhesion proteins is their multi-domain structure. For example, vinculin can interact with at least ten other focal adhesion proteins, including actin, tensin, paxillin, and talin, although probably not simultaneously. In addition to recruiting non-catalytic components, protein kinases also cluster at focal adhesions in response to cell adhesion, which leads to increased phosphorylation of focal adhesion proteins. Paxillin, another important multi-domain adaptor protein, functions in recruiting both signaling and structural molecules to focal adhesions. The COOH terminus of paxil-
AdCC is composed of distinct populations of cells, which can be divided into two types based on ultrastructural and histochemical studies. Type A cells comprise cells of the pseudocyst lining, nonluminal cells, and peripheral cells, and exhibit several myoepithelial markers. Type B ductal or luminal cells express several secretory markers. While type A cells are abundant in tumor tissue, type B cells are rather scarce. Immunohistochemistry studies have shown that type A cells are positive for muscle-specific actin, low-molecular-weight cytokeratin, and S-100, whereas type B cells express carcinoembryonic antigen (CEA), epithelial membrane antigen (EMA), high-molecular-weight cytokeratin. Co-expression of cytokeratin and vimentin has been observed in tumor cells of a variety of salivary gland neoplasms, including AdCC, pleomorphic adenoma, and carcinomas in pleomorphic adenoma. By contrast, human normal salivary glands co-express these proteins, but only in areas where myoepithelial cells and basal cells of the epithelium are present. Nonhomogeneous expression of intermediate filaments in AdCC has also been shown immunohistochemically. Cytokeratin was more striking in luminal cells (type B) of the tumor than in peripheral cells, indicating an inverse correlation between cytokeratin and vimentin expression in AdCC.
the differentiation seen in normal stratified epithelium.

AdCC cells can also be divided into three subgroups histologically: cribriform, tubular, and solid (Fig. 1). The most characteristic pattern is cribriform, created by many pseudocysts scattered in cell islands (Fig. 1C). Perzin et al.\textsuperscript{29} reported that patients whose tumors consisted of the tubular or cribriform pattern had a favorable outcome, in contrast to patients with solid type. Several reports have indicated that the cribriform pattern is associated with the longest patient survival time, followed by tubular and solid types\textsuperscript{1,21,22}. However, other studies were unable to find a relationship between histological pattern and survival, since individual tumors often contained all three cell types\textsuperscript{33}. Perineural invasion may be more associated with an unfavorable outcome (Fig. 1D).

ECM-containing BM components are a histological feature of AdCC. They are observed in the cribriform and tubular patterns of the pseudocyst lining and on the outer surface of cells comprising tubular areas. BM consists of several types of macromolecules, including type IV collagen, laminin, fibronectin, heparin sulfate proteoglycan, and entactin. These proteins have been demonstrated in the ECM surrounding AdCCs, in the pseudocystic spaces, hyaline stroma, and around tumor nests\textsuperscript{34}. Tumor cells appear to proliferate in contact with the BM and to infiltrate through BM-rich tissues (Fig. 2), such as peripheral nerves, blood vessels, and skeletal muscle\textsuperscript{35}. It has been clearly demonstrated that BM molecules in the stroma are produced by the tumor cells themselves\textsuperscript{32} (see sect. 4). Furthermore, the biosynthetic and metabolic changes that occur in the ECM might be involved in malignant transformation.

Many histochemical- and morphological-based observations suggest that myoepithelial-like cells are responsible for the production of ECM components, including BM molecules\textsuperscript{32}. We previously found a correlation between an increase in glycosaminoglycan synthesis and the expression of a myoepithelial-like phenotype in cultured cells\textsuperscript{36}. The production of ECM and BM by AdCC cells is exclusively a myoepithelial or basal cell characteristic, and is vigorous, but disorganized, compared with production in normal tissue.

4. Biological characteristics of cultured cells

1) ECM production and degradation by AdCC-derived cell lines \textit{in vitro} are regulated by the uPA-plasmin system.

Well-characterized cell lines established from human AdCCs, such as Acc-2, Acc-3\textsuperscript{37}, and Acc-M\textsuperscript{38} (established at Shanghai Second Medical University\textsuperscript{39}), ACCS\textsuperscript{39}, ACCY\textsuperscript{32}, and ACCT, serve as useful tools for understanding the biology of this type of tumor. AdCCs vigorously produce and accumulate ECM, including basement membrane components. As it does \textit{in vivo}, the ACCY, ACCS, Acc-2, and Acc-3 cell lines have been shown to produce large amounts of ECM, including collagen, elastin, basal lamina components, and mucopolysaccharides\textsuperscript{1,22,34,40}. The amount of ECM produced by ACCS cells is comparable to that produced by normal mesenchymal cells. ACCS and ACCY cells are of the basal-myoepithelial cell type (see Sect. 2, type A cells) and form pseudocysts \textit{in vitro}. This finding supports suggestions that myoepithelial-like cells are responsible for ECM production, including basal lamina macromolecules, and that the accumulation of ECM in intercellular spaces contributes to the formation of the stroma surrounding AdCCs\textsuperscript{32}.

In addition to extensive ECM-producing activity, ACCS cells can degrade considerable amounts of ECM elaborated by mesenchymal cells, mainly via the uPA-plasmin system\textsuperscript{3}. ACCS cells produce high levels of several proteolytic enzymes, including uPA, MMP-2, and MMP-9 and ECM degradation by the cells is both plasminogen-dependent and closely associated with uPA expression\textsuperscript{3}. \textit{In vitro} study has demonstrated that ECM produced by ACCS cells contains PAI-1 (Fig. 1E) and is consequently resistant to degradation\textsuperscript{3}. As a result, ECM components can accumulate in the intercellular spaces, thereby forming a pseudocyst (Fig. 1C and Fig. 2), which is the characteristic architecture of AdCC\textsuperscript{32}. Since, in this way, AdCC cells are surrounded by their own ECM, as well as by normal host connective tissue, they are able to proliferate along the tumor-produced ECM and then bind to and finally invade adjacent normal tissues (Fig. 2).
2) EGF induces activation of AP-1, which simulates ECM-degrading protease production and migration, while dexamethasone inhibits these events

Certain growth factors, such as epidermal growth factor (EGF), regulate tumor invasiveness by altering cell adhesion, ECM-degrading protease production, and motility. Treatment of AdCC cells and oral squamous cell carcinoma (SCC) cells with EGF stimulates several activities, such as ECM degradation, migration, and Matrigel (artificial basement membrane protein) invasion. In various types of cells, including AdCC and SCC cells, EGF induces increased expression of uPA and uPAR proteins and mRNA through activation of transcription factor activator protein-1 (AP-1). Both EGF-induced invasion and uPA and uPAR expression are markedly inhibited by dexamethasone (DEX) through a reduction of DNA-AP-1 binding and the induction of PAI-1 expression. Rosenthal et al. reported that EGF also stimulates the production of MMP-1, stromelysin-1, and MMP-9 in a process that may be mediated by AP-1. MMPs, particularly MMP-2 and MMP-9, are important for the degradation and invasion of BM and ECM. Initially, MMPs released from cells are inactive; they are converted to active forms by other MMPs or plasmin. Since uPA is activated by binding with uPAR, the uPA-uPAR interaction is essential for augmenting proteolytic activity and uPAR-mediated signaling, which ultimately induce motility and invasion. DEX inhibits the expression of both uPA and uPAR by blocking AP-1 activation, and therefore may be useful in the treatment of malignant tumors.

3) AdCC migrates to types I and IV collagens

As noted above, due to its proclivity for invading nerve and endothelial sheaths, the frequency of recurrence and distant metastasis of AdCC is very high. Data from our laboratory suggest that AdCC cells initially proliferate along tumor-produced ECM, containing BM components, and that these invasive cells adhere and migrate to nerve and endothelial sheaths, which also contain BM components. In fact, ACCS-produced ECM strongly stimulates the migration of these cells in vitro. Moreover, the ability of ACCS and ACCT cells to migrate and adhere to ECM, especially types I and IV collagen, is greater than that of oral SCC cell lines, although AdCC and SCC cells have very similar profiles of integrin subunit expression. The collagen-stimulated migration of AdCC cells is largely and exclusively inhibited by anti-α2 integrin antibodies, suggesting that α2 integrin is functionally important for the adhesion and migration of AdCC cells to collagen.

4) AdCC cell migration to collagens may be due to uPAR overexpression, which plays a key role in focal-adhesion assembly and migration

Various integrin-associated proteins have been reported to modulate integrin signaling and to control integrin-mediated events. The multifunctional surface receptor uPAR is one such protein that controls signaling, adhesion, and migration. Compared with normal cells, malignant tumor cells usually express higher levels of uPA and uPAR, which results in increased tumor cell invasion in vitro. The results of our studies showed that AdCC cells produce a large amount of uPA and degrade ECM via the uPA-plasmin system, and that uPAR surface protein and mRNA expression are greater in AdCC than in SCC cell lines. Combined, these findings suggest that the characteristic properties of AdCC cells, invasion, and metastasis, are mainly regulated by the uPA-uPAR system. In this respect, uPAR has been shown to facilitate cell-surface plasminogen activation, which generates a proteolytic cascade contributing to matrix degradation during tumor invasion, and might function in cell adhesion and migration. Several studies have demonstrated the presence of uPAR at focal adhesion sites and at leading edges of migrating cells, suggesting that uPAR physically associates with integrins. Figure 4B shows a cell clone transfected with pECF-N1 vector containing uPAR cDNA and cDNA for the enhanced cyan fluorescent variant of green fluorescent protein (CFP). CFP-uPAR overexpression resulted in an increased frequency of the formation of lamellipodia, in which CFP-uPAR was concentrated. This finding indicates that receptor expression plays a role in actin reorganization, which is regulated by genes of the Rho family. When AdCC cells were plated on collagen, the surface level of uPAR increased, and numerous focal adhesions, consisting of uPAR, vinculin, and paxillin, were assembled (Fig. 4A, C and D), whereas collagen-stimulated SCC cells or AdCC cells plated on other types of ECM, such as fibronectin, failed to assemble focal adhesions. Figure 4C shows a case
of comparison of a transfectant of ACCS with uPAR antisense (ACCS-AS) and control clone (ACCS-zeo). The surface uPAR level of AdCC cells was up-regulated by collagen stimulation in the absence of uPAR mRNA induction\(^39\). As shown in experiments with EGF, uPAR by collagen stimulation in the absence of uPAR mRNA expression is controlled mainly at the transcripational level\(^44\). Post-transcriptional regulation and recycling of uPAR to the plasma membrane represent additional levels of regulation\(^7\). In a study applying immunocytochemical staining, we demonstrated the translocation of uPAR from the cytoplasm to focal adhesions on collagen stimulation of AdCC cells, while SCC cells expressing low levels of uPAR were less able to assemble focal adhesions. In AdCC cells, uPAR co-localized with \(\alpha_\beta 3\) integrin, paxillin, and vinculin at focal adhesions following collagen (Fig. 4C and D), but not fibronectin, stimulation\(^39\). These findings suggest that on collagen activation, uPAR migrates to the focal adhesion via its interaction with a collagen receptor, probably the \(\alpha_\beta 3\) integrin receptor. Recent reports indicated that uPAR, which is a GPI-anchored protein, plays an important role in regulating cell migration via the modification of actin-integrin adhesive complexes\(^6,55,57,59,60\).

The down-regulation of uPAR inhibits the growth and migration of carcinoma cells in vitro\(^61,62\), alters cytoskeletal organization in human glioma cells\(^63\) and fibroblasts\(^64\), and inhibits colon cancer metastasis in a nude mouse model\(^65\). However, how down-regulation of uPAR...
inhibits invasion and metastasis is poorly understood. Our immunoprecipitation experiments showed novel interactions between $\alpha_2\beta_1$ integrin or paxillin and uPAR in AdCC cells induced by type I collagen stimulation. Furthermore, down-regulation of uPAR led to conformations changes in focal adhesions and actin organization. 

Reduced stress fibers and paxillin were strongly reduced in ACCS-AS cells. uPAR expression in these cells is about 10% that of parental ACCS cells at both the protein and mRNA levels. Collagen-stimulated migration and focal adhesion assembly of $\alpha_2$ integrin, vinculin, and paxillin were strongly reduced in ACCS-AS cells. Moreover, down-regulation of uPAR led to conformational changes in focal adhesions and actin organization (reduced stress fibers). The expression and distribution of $\alpha_2$ integrin were obscured in ACCS-AS cells plated on any ECM, and there were fewer focal adhesions, as shown by the staining pattern for paxillin. This suggests that uPAR plays a role in regulating the recruitment of $\alpha_2$ integrin to focal adhesions, and in their assembly, and that migration of AdCC cells to types I and IV collagens is due to the overexpression of uPAR.

5) Acc-M is a model cell line for examining the mechanism of lung metastasis

In contrast to oral SCCs, which metastasize lymphatically, AdCCs undergo blood-borne metastasis and in most cases metastasize to the lungs. Although the underlying molecular mechanisms are complex, it is clear that metastasis requires a cascade of sequential steps involving the coordination of adhesion, motility, and growth. After tumor cells intravasate into the bloodstream, they evade innate immune surveillance, adhere to vascular endothelial cells of distant organs, and extravasate into those tissues. Subsequently, the malignant cells settle in the parenchyma of target organs where they create metastatic deposits.

The well-characterized lung metastatic AdCC cell line Acc-M was established by repeated isolation from lung metastatic foci of Acc-2 cells. Cloning of the cells revealed a close relationship between metastasis and platelet aggregation activity. Acc-M-GFP expresses green fluorescence protein (GFP) and is useful for the visualization of metastatic foci in vivo. Injection of Acc-M-GFP cells into the tail vein of athymic nude mice resulted in metastasis to multiple sites, including lung, muscle, bladder, and bone. This experiment attempted to model the events that occur after tumor cells intravasate into the bloodstream. We showed that the adhesion of Acc-M cells to human umbilical vein endothelial cells is greater than that of non-metastatic AdCC cells, and that the interaction between tumor cells and vascular endothelial cells is mediated through adhesion molecules, including E- and P-selectins. These are expressed on stimulated endothelial cells and activated platelets and react with selectin glycoprotein ligand, which mediates leukocyte rolling on stimulated endothelial cells and the heterotypic aggregation of activated platelets on leukocytes. A recent study suggested that P-selectin binds to cancer cells, including Acc-M, in vitro and promotes the growth and metastasis of human carcinomas in vivo and sulfation was also shown to be essential for Acc-M cell adhesion to P-selectin.

Although the molecular mechanisms of AdCC cells are complex, it seems likely that metastasis is regulated by interactions of adhesion molecules and proteases. Further studies are required to resolve the complex mechanisms.

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